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Ixodes Scapularis SRC Kinase Is Required for Rickettsial Pathogen Survival in Ticks

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***IXODES SCAPULARIS* SRC KINASE IS REQUIRED FOR RICKETTSIAL
PATHOGEN SURVIVAL IN TICKS**

by

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A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
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ABSTRACT

IXODES SCAPULARIS SRC KINASE IS REQUIRED FOR RICKETTSIAL PATHOGEN SURVIVAL IN TICKS

Jeremy W. Turck
Old Dominion University, 2020
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Anaplasma phagocytophilum is an obligate intracellular bacterium that causes disease in humans and animals. It is the causative agent for human anaplasmosis. *A. phagocytophilum* uses certain strategies to infect both vertebrates and invertebrates. It uses *Ixodes scapularis* ticks as a vector for spreading infection to other mammal species. This bacterium has a specific path for infection through the salivary glands of its vector host. It also suppresses certain functions such as the inhibition of apoptosis and ROS production in order to increase its survival in ticks. Src kinase, a non-receptor protein-tyrosine kinase, is a major player in cell signaling. Src kinase has an impact on the spread and survival of *A. phagocytophilum* in ticks. Studies on Src kinase in *I. scapularis* show that there is a downregulation of Src in unfed ticks and an upregulation after tick feeding. These results show that Src kinase is manipulated differentially by this bacterium for its survival in the vector host and during transmission from vertebrate host to ticks. Furthermore, RNAi-mediated interference of arthropod *src* kinase gene expression or inhibition of Src kinase activity by inhibitor treatment resulted in dramatic reduction in bacterial loads in ticks and tick cells. Collectively, the findings from this study show that Src kinase plays a major role in tick-*A. phagocytophilum* interactions. Understanding the role of Src kinase in tick-*A. phagocytophilum* interactions could lead to identification of vaccine targets that eventually could lead to preventing the spread of this disease.

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Nomenclature

Ats-1	<i>Anaplasma</i> translocated substrate
AnkA	Ankyrin Repeat Domain containing Protein A
cDNA	Complementary Deoxyribonucleic Acid
DF	During Feeding
DNA	Deoxyribonucleic Acid
ERK	Extracellular signaling-regulated kinase
FAK	Focal Adhesion Kinase
HGE	Human Granulocytic <i>Ehrlichiosis</i>
JAK	Just Another Kinase
I	Infected
MAPK	Mitogen Activated Protein kinase
PCR	Polymerase Chain Reaction
PF	Post Feeding
PKA	Protein Kinase A
PKC	Protein Kinase C
qPCR	quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RT-qPCR	Reverse Transcriptase – quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
STAT	Signal transducer and Activator of Transcription
UI	Uninfected

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CHAPTER I

INTRODUCTION

I. Anaplasmosis

Anaplasma phagocytophilum is a zoonotic pathogen that is able to infect both vertebrates and invertebrates. This pathogen is an obligate intracellular bacteria that is primarily transmitted through *Ixodes* species of ticks (Alberdi et al., 2016). *A. phagocytophilum* causes tick-borne fever and granulocytic anaplasmosis in humans and animals (Kocan et al., 2015). This bacterium is gram negative, has a coccoid morphology, and mainly attacks immune cells, specifically neutrophils and endothelial cells (Alberdi et al., 2016). This bacterium has certain strategies to avoid host immune responses and to ensure its survival in the tick vector.

II. *Ixodes*

The life cycle of *Ixodes scapularis* ticks goes through several stages in order for it to develop and reproduce. Adult female ticks will lay eggs, which will hatch into the larval stage of the tick. The larvae will then attach to a smaller mammal, such as mice or rat, and will then take a blood meal from the host. The larvae will detach from the host after engorgement and will then molt and develop into the nymphal stage of tick. Nymphs are larger in size and can attach to larger animals such as deer and dogs. The nymphs will then take a blood meal when they are attached to a host and will detach after engorgement. The nymphs will then go through another molting phase and will then develop into an adult male or female. Adult female feed on larger mammals and they mate with male ticks. The female ticks will lay eggs and this will return back to the beginning of the life cycle (Khanal et al., 2018)

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When a tick feeds on a host infected with the pathogen *A. phagocytophilum*, the tick vector has the possibility of ingesting this bacterium while it is taking a blood meal. Upon entering into ticks, the bacteria will migrate to the midgut of the vector where certain factors will guide it to the salivary glands of the vector. When the vector takes a blood meal from a host it has the possibility of transmitting the bacteria in its saliva to the host. There are several hosts that can be infected by this pathogen and could serve as reservoir hosts which help contribute to its spread and survival (Kocan et al., 2015). When the bacterium enters the host circulatory system, it will then employ factors that will help it to escape from host immune response by delaying apoptosis of neutrophils. In the tick gut, the pathogen is able to manipulate apoptosis through the JAK/STAT pathway which could lead to the transcription of apoptotic factors (Alberdi et al., 2016). This in turn could aid in its infectivity and support its survival in host cells.

III. Signal transduction

A. phagocytophilum has evolved to avoid forms of destruction within cells. When this pathogen is inside a cell, it will form a dense intracellular colony within a vesicle called morulae. The pathogen will stay within vacuoles in the cytoplasm of the cell in both vertebrate host and the vector (Kocan et al., 2015). Within the cell, it is able to divide and reproduce while secreting factors to prevent the cell from interacting and destroying these structures. *A. phagocytophilum* has a type IV secretion system and one form of host invasion is the secretion of Ats-1 (Alberdi et al., 2016). This factor translocates to mitochondria and prevents apoptosis. Apoptosis prevention can be done through the inhibition of mitochondria-mediated apoptotic caspases. There are several caspases associated with the initiation and regulation of apoptosis within the cell. *A. phagocytophilum* excretes factors that cause the delay of this process aiding in its ability

to reproduce within the cell. Certain signal transduction pathways such as JAK/STAT are utilized to influence regulatory functions such as apoptosis, actin modulation, and reactive oxygen species (Alberdi et al., 2016). In vertebrates, B and T cells have an important role in the clearance of *A. phagocytophilum*. In mice, the infection is usually cleared in about 10 to 12 days (Sukumaran et al., 2012).

Another article showed that *Anaplasma phagocytophilum* induces phosphorylation of actin. This finding shows that *A. phagocytophilum* influences cytoskeleton remodeling and has an effect on signal transduction (Sultana et al., 2010). This pathogen can cause a cascade of effects that would impact the gene expression of certain cellular factors associated with reproduction and regulation. It is important to understand the events that cause these events from happening and in order to do this there needs to be a working model of the signal transduction pathway for this pathogen. Tyrosine phosphorylation plays an important role in this pathway and is a way for this pathogen to increase its survival within the cell (Sultana et al., 2010). This pathogen influences actin cytoskeleton structures through the secretion of certain factors that take advantage of the intracellular signaling in the cell (Sultana et al., 2010). Utilizing the proteins within the cell aids *A. phagocytophilum* in its infectivity and survival.

IV. Src Kinase

A protein that plays a role for the internalization of intracellular pathogens is Src kinase. Src kinase is a non-receptor tyrosine kinase and is located in the intracellular space of a cell. This protein has an important role with the internalization of other bacterial pathogens and when it is inhibited, there is a decreased ability for pathogens to survive within the cell (Wu et al., 2011). Src can be phosphorylated or dephosphorylated by other proteins and Src kinase can also phosphorylate other protein structures (Ijdo et al., 2007). This shows that Src kinase has an

effect on several proteins which would affect downstream signaling within the cell. Src kinase is activated through dephosphorylation and exists in its closed inactive form when it is phosphorylated (Copiz et al., 2016). There are proteins that can activate and deactivate Src kinase such as FAK, PKA, and PKC and this in turn will have an effect on cell survival, apoptosis, cell proliferation, oxidative stress, and cell differentiation (Copiz et al., 2016). *A. phagocytophilum* takes advantage of the cell in order to manipulate these functions in order to aid in its own survival. Src kinase has an enormous impact on intracellular cell regulation and maintenance. This protein needs to be understood in order to find out the specific role it has on the signaling transduction pathway within cells. Also looking into this pathway could aid with vaccine development and more methods for detection of this pathogen.

V. Src interactions

Src has several intracellular functions and Figure 1 outlines the some of the known details about Src kinase signal transduction (New et al., 2007). Figure 1 shows how Src uses JAK/STAT and PKC in order to regulate transcription factors that are needed to regulate the cell cycle (New et al., 2007). Src can cause complexes to form that would cause certain genes to be transcribed. Figure 2 goes more in depth on how Src kinase has influence on cell regulation and how it can be affected and affects other proteins (Westhoff et al., 2004). In figure 2, it shows that Src kinase is dephosphorylated and is in its active state where it can then phosphorylate other proteins or kinases can cause a cascade of effects that lead to cell growth and survival (Westhoff et al., 2004). This shows that Src kinase plays an important role with the ability of cells to reproduce, assemble, and survive.

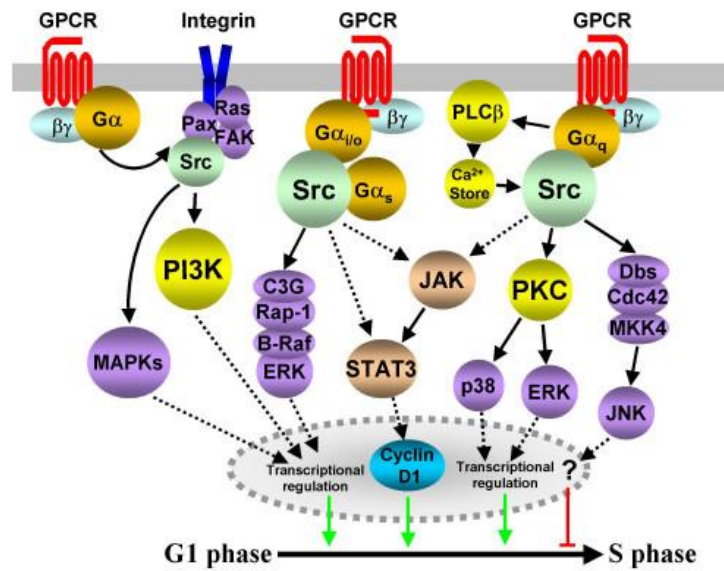


Figure 1. Src Kinase Model of the Intracellular Pathway Mechanisms.

This figure represents the signal transduction cascade of Src kinase on the intracellular side of a cell and shows the effects that it has on cell cycle (New & Wong, 2007). Permission granted by Creative Commons License: <http://creativecommons.org/licenses/by/4.0/>

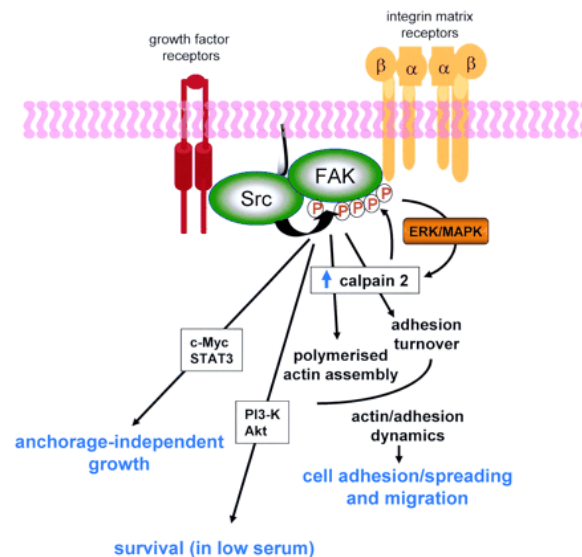


Figure 2. Src and FAK Model for Interaction and the Downstream Effects.

This figure shows the kinases Src and FAK on the intracellular side of the cell membrane and illustrates the ability of Src kinase to influence other protein structures and factors that influence cell structure and cell cycle (Westhoff et al., 2004).

CHAPTER II

METHODS

I. Sample preparation

Samples were collected through the homolysis of tick or tick cell samples. Tick samples were ground in an RNA lysis solution from an Aurum total RNA mini kit (Bio-RAD, USA). After the samples were ground, they were centrifuged and the supernatant was collected. The lysate underwent RNA isolation with the use of a Total RNA extraction BioRad kit. The purified and concentrated RNA samples were used in a reverse transcriptase reaction using protocols and materials from a Bio-RAD iScript cDNA synthesis kit (Bio-RAD, USA), to produce the cDNA samples. Generated cDNA samples served as the template for the Src transcript quantification and this was used in the RT-qPCR reactions. These reactions also used the forward oligonucleotide 5' CGCGCACGGACGAGGA 3' the reverse oligonucleotide 5' GTTCTGCCTCGATGGACTTCAGT 3' as the primers which were included with the RT-qPCR reactions as shown in Table 1 below. These reactions also included an intercalating agent for the detection of the amount of DNA in each sample. The intercalating agent was a SYBR green supermix from BioRad. The RT-qPCR used absolute quantitation for the analysis of each sample. The *in vitro* studies with the tick cell samples underwent a similar process. The tick cells were plated and given 24 hours of incubation in order to adhere to the plates. Upon collection the supernatant of the tick cells were removed and an RNA lysis solution from a Bio-Rad kit was added to each well. The tick cells then underwent the RNA isolation protocol and then the cDNA synthesis protocol from the respective BioRad kits and were subsequently used in the RT-qPCR.

Table 1. Oligonucleotide Sequences that were Used for Src Kinase Production and dsRNA Production of Src Fragment.

Oligonucleotide Sequences	
DNA Component	Sequence
Src Kinase Amplification	
Forward	5' CGCGCACGGACGAGGA 3'
Reverse	5' GTTCTGCCTCGATGGACTTCAGT 3'
Src dsRNA Amplification	
Forward	5' CCAGATCTCGAGCTCCAAGAACACCAAGA 3'
Reverse	5' CGGGTACCGCGCGAACCACCAGT 3'

II. Mice and bacteria

Mice were used in these experiments and the mice work was done with accordance with the regulations of the Care and Use of Laboratory Animals of the National Institute of Health. The Institutional Animal Care and Use committee (IACUC) approved the protocol used in the study. The IACUC Animal Welfare Assurance number was A3172-01 and the protocol permit number was 16-017. Mice were either uninfected or infected with *A. phagocytophilum*. C3H/HeN female mice, 4-6 weeks old, were used in this study (Turck et al., 2019). A protocol was used for the isolation of *A. phagocytophilum* from HGE mammalian cells. This protocol consisted of a series of passages through a needle in order to rupture the cells and release their

contents and then separating the cell debris from the mixture (Sultana et al., 2010; Turck et al., 2019). Infected mice were injected with 100 microliters of the isolated *Anaplasma*.

Anaplasma solution was isolated from infected HL-60 cells. *A. phagocytophilum*-infection of ticks or tick cells was performed based on approved IACUC protocol 15-012. The HL-60 cells were placed in a flask and were allowed to grow. The HL-60 cells were infected with *A. phagocytophilum*. The *I. scapularis* tick cell line ISE6 and *A. phagocytophilum* strain HGE1-GFP was obtained from Dr. Munderloh at the University of Minnesota (Sultana et al., 2010; Turck et al., 2019). HGE1-GFP and HZ strains of *A. phagocytophilum* were used in this study. The cell culture was taken and centrifuged to isolate bacteria as described (Sultana et al., 2010; Turck et al., 2019). The sample was centrifuged and the *Anaplasma* was collected from the supernatant and this was used to infect naïve cells or mice.

III. RT-qPCR

RT-qPCR was done on the samples generated from different life cycle stages, including larvae, nymphs, adult males, and adult females. To generate unfed nymphs, larvae were fed on uninfected or *A. phagocytophilum*-infected mice and were then molted. During feeding tick samples were generated from ticks that were allowed to feed on the host for 48 hours, then were removed for analysis. To generate post-fed ticks, unfed larvae or nymphal ticks were fed on host to complete engorgement. After 48 hours post-repletion, ticks were processed for RNA, DNA or protein extraction. Samples used in this study were generated either from uninfected or *A. phagocytophilum*-infected ticks.

Primers were designed for the amplification of tick actin, tick Src kinase, and p44 (Turck et al., 2019, Taank, et al., 2017). p44 was used for the detection of *Anaplasma* DNA in samples and the tick actin gene was used as a housekeeping gene for comparison and normalization of the

Src kinase expression and evaluation of bacterial burden. The absolute quantitation was found through the fluorescent detection of SYBR green by the qPCR unit software. The absolute quantitation was able to give the amounts of DNA or RNA present in the samples based on the amount of fluorescent detection and through the use of standards. The standards were generated through the amplification of the cDNA and were quantitated with the use of a TECAN Infinite M200 Microplate Reader (TECAN, USA). The TECAN instrument measures the density of the solution using UV detection and provided the density of each standard solution. The software program used the data from the standards to produce the absolute quantitation of DNA of each unknown sample that was placed into the wells of the qPCR plate. Standards were made for tick actin, tick Src kinase, and p44 and had a 10-fold serial dilution. Tick actin was used as the housekeeping gene for comparison for both the Src kinase and p44 and had a standard curve ranging from 1 nanogram to 0.00001 nanogram.

IV. Western Blotting

The samples used for immunoblotting were from the total lysates of uninfected or *A. phagocytophilum*-infected unfed and fed ticks or ISE6 tick cells. The tick cells were collected in a RIPA buffer with an EDTA-free protease inhibitor and phosphatase inhibitor solution. Primary and Secondary antibodies were purchased from Santa Cruz Biotechnology Inc. Protein concentration was measured using a Bradford (BCA) Protein Assay kit and 30 micrograms of protein sample was used for the unfed samples and 10 micrograms of the fed sample was used for the immunoblotting. SDS-PAGE gels were used for immunoblotting and Ponceau and Coomassie Brilliant Blue stained gel images were used to show total protein profile. A WesternBright ECL kit was used for the detection of antibody binding and the blots were imaged with a Chemidoc MP imaging unit.

V. dsRNA synthesis

Primers with BglII and KpnI restriction enzyme sites were designed for generating the *src*-dsRNA fragment of 334 base pairs length (bp). A KpnI site was present at 187 bp position. Upon digestion with BglII and KpnI, two bands were obtained. A 186 bp fragment that contained BglII and KpnI sites that was used for cloning into pL4440 vector. Primers were used for the generation of the clone fragment that had the forward oligonucleotide sequence 5' CCAGATCTCGAGCTCCAAGAACACCAAGA 3' and the reverse oligonucleotide sequence of 5' CGGGTACCGCGCGAACCACCAGT 3', as shown previously in Table 1. The fragment that was amplified was purified using a gel extraction kit from QIAGEN and the purified Src fragment sample was cloned into the pL4440 plasmid vector. *Escherichia coli* DH5α cells were used as the host for generating the clone. Plasmid extractions were performed from bacteria containing cloned plasmid. The plasmid extracts were used to produce the dsRNA through the use of a MEGAscript RNAi kit (Ambion, USA).

VI. Microinjected ticks

Microinjections were performed as described (Khanal et al., 2018; Taank et al., 2017; Turck et al., 2019). The dsRNA experiment included mock and *src*-dsRNA-treated ticks. The *src*-dsRNA-treated tick were generated by microinjecting purified dsRNA and the mock ticks were injected with elution buffer or fragment generated from pL4440 vector. Unfed nymphal ticks were used for the microinjection experiments. Ticks were placed on the mice 4 hours after the microinjection of *src*-dsRNA. The ticks were placed onto infected mice and were collected after they had fallen off of the host. The ticks that were collected were homogenized 48 hours after they had fallen off of the mice. Homogenized tick samples were processed for RNA, DNA, and protein extractions.

VII. dsRNA transfection into tick cells

Tick cells were plated onto the wells on a 12 well plate and were allowed 24 hours to adhere to the plate and incubate. Each plate had 1.0×10^5 tick cells per well. dsRNA was incorporated into the tick cells with a lipofectamine transfection reagent and this was used to introduce the dsRNA into the tick cells. The tick cells were either treated with dsRNA or treated with the mock solution. The treated tick cells had 500 micrograms of *src*- dsRNA mixed with a lipofectamine reagent. The mock tick cells had equal volume of elution solution added into the wells. The elution solution was the same one that was used for eluting the dsRNA. The dsRNA was mixed with the lipofectamine reagent and was added to the plate wells. After 6 hours a 2X L15-B300 media was added to the plate wells. Then 24 hours after transfection, all of the tick cell wells were infected with *A. phagocytophilum*. After 24 hours of infection the cells were collected in lysis solution for RNA or DNA extractions.

VIII. Tick cell inhibitor studies

A Src kinase inhibitor that can inhibit both Src and Lck was obtained from Cayman Chemicals. A 5 millimolar stock was made in DMSO and the concentration used in the experiments was 1 millimolar. Tick cells were plated onto 12 well plates with 1.0×10^5 cells per well and were allowed to incubate for 16-20 hours. DMSO was considered as mock solution. Experimental wells had a 5 micromolar concentration of the inhibitor. Tick cells were treated for 4 or 24 hours before being challenged with *A. phagocytophilum*. Src transcript levels and *A. phagocytophilum* loads were analyzed as described in other sections.

CHAPTER III

RESULTS

I. Tick *in vivo* studies

The results from figures 3 and 4 represents the RT-qPCR data that was produced from RNA samples generated from different life cycle stages of ticks. These are *in vivo* tick samples that show the association of Src kinase signal transduction pathway with *A. phagocytophilum* infection. Figure 3 shows data that represents the different stages of development for ticks. With Figure 3A, the data shows that there is a significant difference in Src kinase gene expression with larvae, nymph, adult female, and adult male. The difference between all of these stages was a significant difference with a p-value less than 0.05. Five individual tick samples were used for the experiment in Figure 3A and all of the ticks were uninfected samples. Figure 3B shows that there is a significant difference with the unfed nymphs when *Anaplasma phagocytophilum* infection is introduced into the ticks. Five independent tick samples were used for the experiment in Figure 3B and it shows that there is a significant decrease in Src kinase expression when the ticks are infected. Figure 3C shows the immunoblotting for the uninfected unfed nymph samples and the infected unfed nymph samples. Figure 3C shows that there is a decrease in the Src kinase protein levels in *A. phagocytophilum*-infected tick samples and this difference can be seen around the 60 kDa marker on the blot. Figure 3D shows src transcript levels in ticks during feeding and the data shows that upon *Anaplasma* infection there is a significant decrease in Src kinase expression.

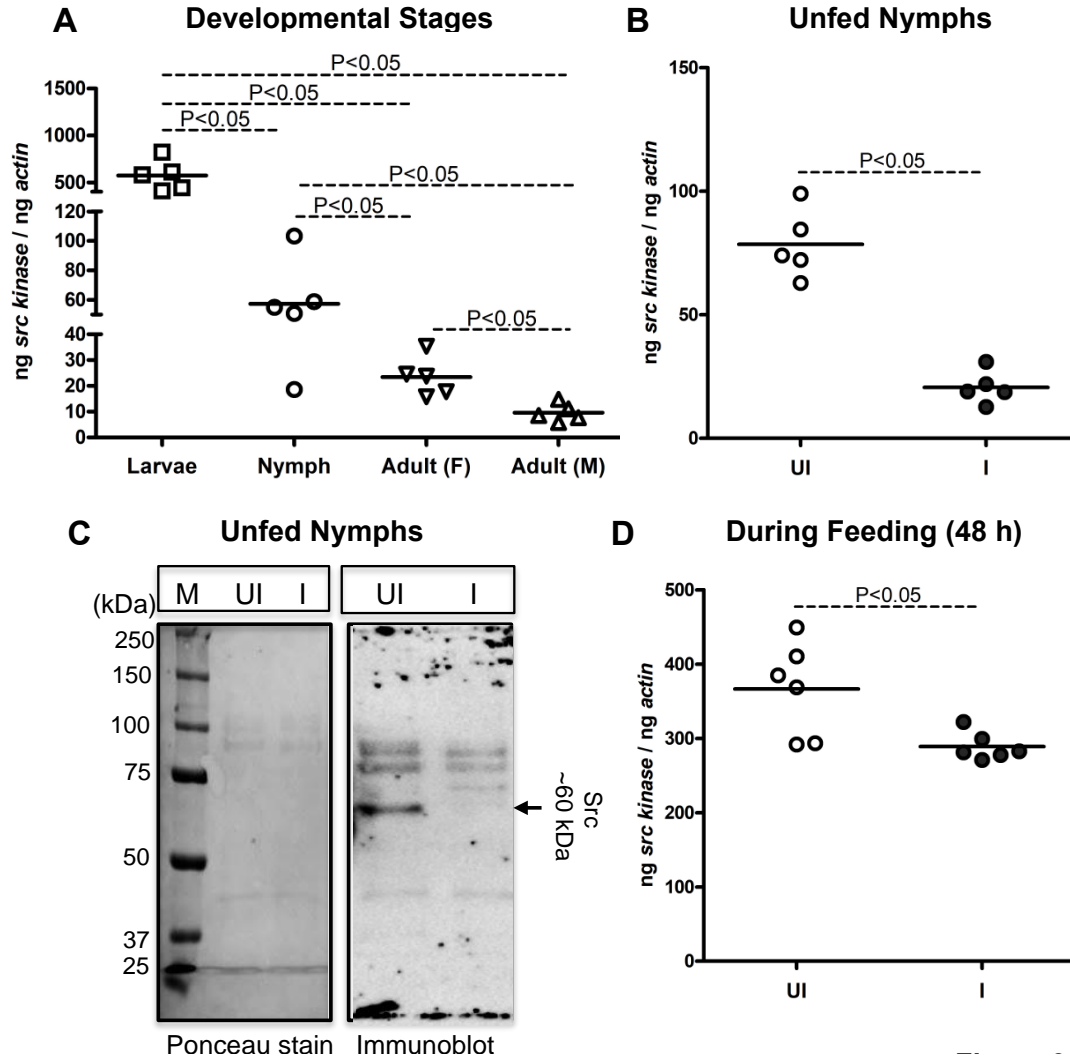


Figure 3. Gene Expression in vivo Analysis for Various Tick Samples.

- (A) RT-qPCR *src* gene expression for different tick life cycle stages.
 (B) *Src* kinase expression in uninfected and infected unfed nymph tick samples.
 (C) Western blot imaging of unfed nymph *Src* kinase protein samples.
 (D) *Src* kinase expression in during feeding nymph tick samples.

Figure 4 displays the results obtained with ticks that have completed the feeding process and have acquired a full blood meal from the host. Figure 4A shows the data for fed larvae tick samples. It demonstrates that there is a significant upregulation in *src* kinase transcript loads in these samples upon *Anaplasma* infection. Figure 4B further builds upon the results of Figure 4A that shows the same trend in fed nymphal tick samples. Increased *src* transcript levels were

noted in *A. phagocytophilum*-infected fed nymphal tick samples. Figure 4C is an immunoblot image showing that there is an upregulation of the expression of the Src protein in *A.*

phagocytophilum-infected samples. Figure 4C shows a difference between the two bands around 60 kDa where the infected tick samples have a darker band showing an increase in the Src protein amounts in this sample. The Ponceau stained image that shows total protein profile was used as the control image for immunoblotting analysis.

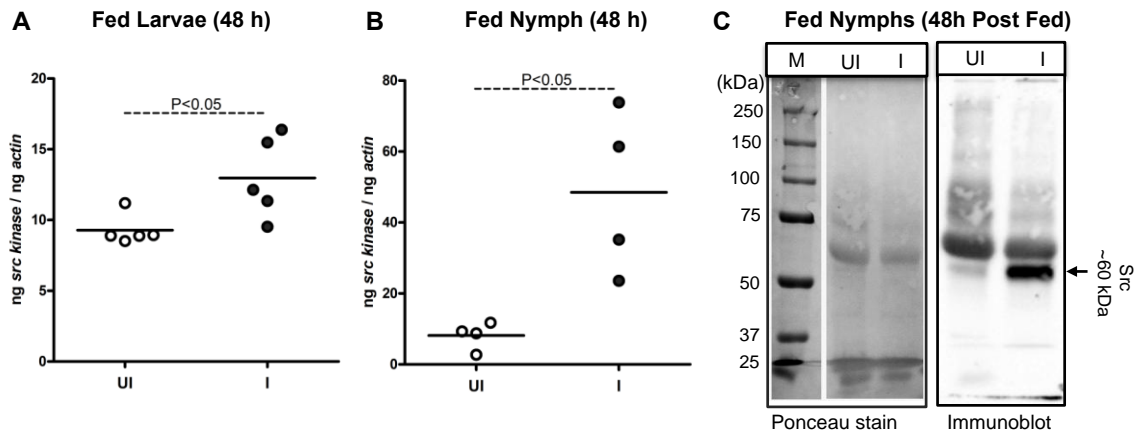


Figure 4. Gene Expression in vivo Data for Fed Nymph Tick Samples.

(A) Src kinase expression in fed larvae samples using RT-PCR.

(B) Src kinase expression in fed tick nymph samples.

(C) Western blot of analysis of Src kinase protein with fed nymph tick samples.

II. Tick *in vitro* studies

Figure 5 shows data on the effects of increased infectivity in tick cells. This experiment is an *in vitro* experiment using ISE6 tick cells where the tick cell samples were challenged with varying amounts of isolated *Anaplasma*. Different amount of *A. phagocytophilum*-culture (100, 200, 400 microliters) was used for infection of tick cells. Bacterial loads in Figure 5A was determined by monitoring the *A. phagocytophilum*-P44 DNA levels. With Figure 5A it can be seen that the level of bacteria increases as the amount of *Anaplasma* solution added to the tick

cells increase. This figure shows that the samples used had an increased infectivity associated with them as the amount of isolated *Anaplasma* solution increased. The differences between the uninfected and infected samples are significant for each category. Figure 5B is observing the effects of increased *Anaplasma* burden on Src kinase expression. As the amount of isolated *Anaplasma* solution increases there is an increase in the gene expression of Src kinase. Figure 3B also shows that each of the infected tick cell categories had a significant upregulation in the expression of Src kinase when compared to the uninfected samples. Figure 5C is looking at the protein amounts in these samples. The immunoblotting results show an increase in the amount of Src protein levels (~60 kDa) with the increase in *Anaplasma* loads. A coomassie blue stained image was used as the control to show total protein profiles.

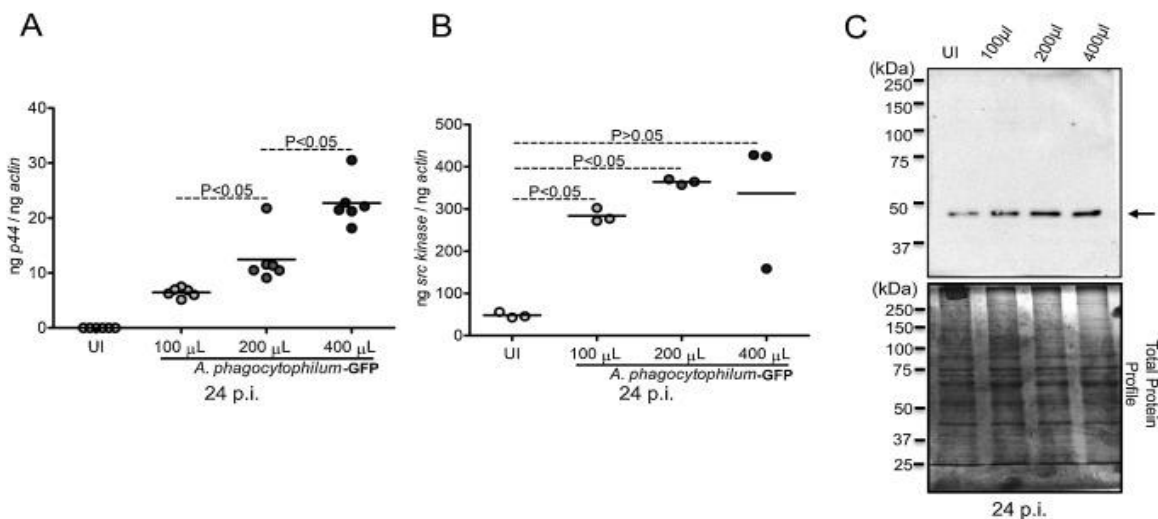


Figure 5. In vitro Data for the Dose Response from ISE6 Tick Cells Infection with *A. phagocytophilum*.

(A) p44 expression in tick cells with varied amounts of isolated *Anaplasma* solution. (B) Src kinase expression in tick cells with varied amounts of isolated *Anaplasma* solution. (C) Western blot imaging of Src kinase protein with dose response tick cell samples and Coomassie Blue stain protein profile of dose response tick cell samples.

III. Tick dsRNA and inhibitor studies

Figure 6 is showing the knockdown studies and the inhibitory studies of Src kinase. Figure 6 includes both *in vitro* and *in vivo* studies using microinjected unfed nymphal ticks and ISE6 tick cells. Figure 6 shows the effects of knocking down or inhibiting Src kinase and the effects that occur during *A. phagocytophilum* infection. Figure 6A, shows *src* gene expression silencing efficiency in *A. phagocytophilum* infected ticks upon treatment with *src*-dsRNA. The results show that there is a significant decrease in *src* transcripts in *src*-dsRNA-treated ticks in comparison to mock-treated control. The same trend can be seen with Figure 6B, significantly reduced bacterial loads were observed in *src*-dsRNA –treated ticks in comparison to mock-treated ticks. Figure 6C shows the effects of *A. phagocytophilum* infection on *src* expression in ISE6 tick cells at different time points of infection. There is a significant difference in *src* transcript levels between the uninfected and infected samples at both time points (24, 48 p.i.). Figure 6D shows the silencing efficiency in ISE6 tick cells and there is a significant decrease in *src* expression between the mock and the *src*-dsRNA-treated tick cells samples. Figure 6E shows reduced *A. phagocytophilum* burden in *src*-dsRNA treated tick cells in comparison to mock-treated cells. Figure 6F shows the effects of the Src inhibitor in tick cells. Figure 6F shows the results of 4 hours of Src inhibition before infection and 24 hours of Src inhibition before infection. In the experiment the tick cells were infected with *A. phagocytophilum* for 24 hours after Src inhibition for certain amount of time. The results of Figure 6F shows that there is a decrease in *A. phagocytophilum* detection in the samples that were generated from tick cells treated with Src inhibitor in comparison to the bacterial loads noted in mock control. There is a significant difference in *src* expression in Figure 6C at both 24hour and 48 hour time points.

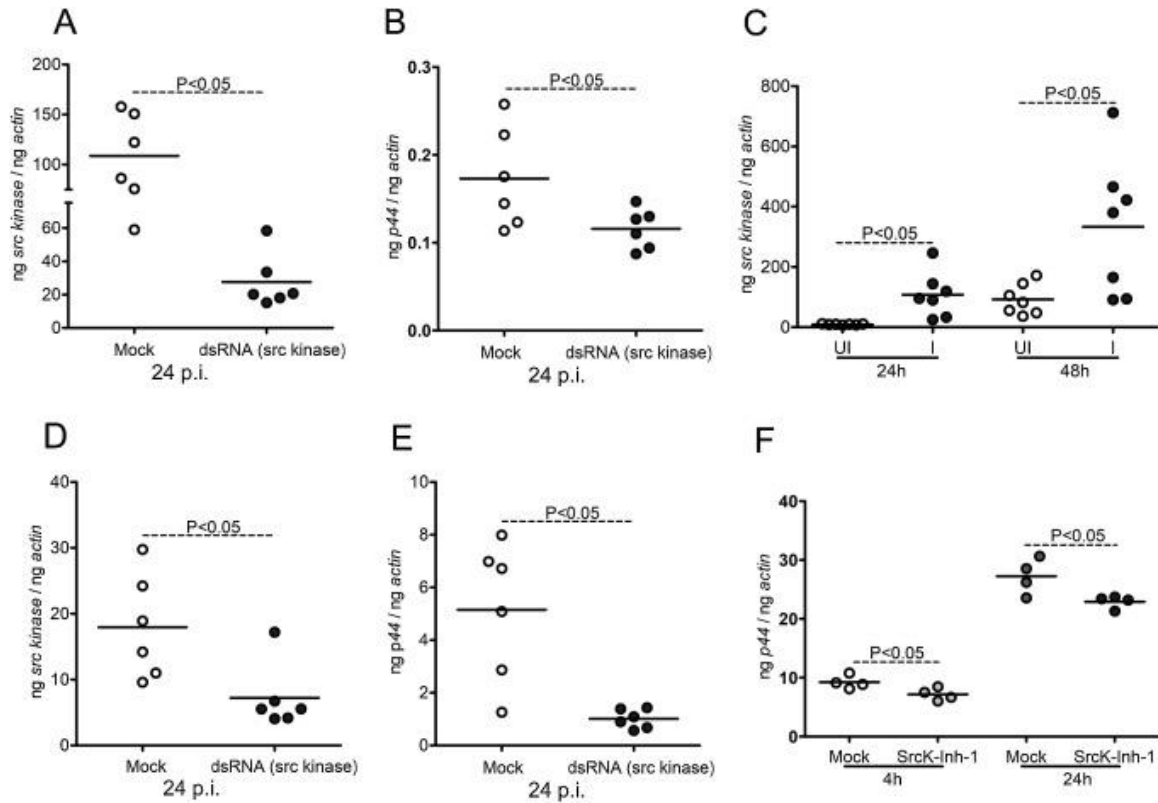


Figure 6. Gene Knockdown in vivo and in vitro Data for Tick and ISE6 Tick Cell Samples.

(A) Src kinase expression in mock and dsRNA microinjected ticks.

(B) p44 expression in mock and dsRNA microinjected ticks.

(C) Src kinase expression in uninfected and infected tick cells with either 24 hours or 48 hours of *Anaplasma phagocytophilum* infection.

(D) Src kinase expression in mock and dsRNA treated tick cells.

(E) p44 expression in mock and dsRNA treated tick cells.

(F) p44 expression in Src inhibited tick cells with either 4 hours or 24 hours of Src inhibition.

CHAPTER IV

DISCUSSION

I. Tick *in vivo* analysis

Figure 3A shows that there is a decrease in Src kinase amounts as tick mature. This data suggests that ticks are less dependent on higher amounts of Src kinase as they molt into their more mature life cycle stages. This is most likely due to the amounts of growth and differentiation that occurs at earlier stages of tick life and less of a need as time goes on. Figure 3B shows that unfed nymphal ticks that have not yet taken a blood meal have decreased amounts of Src kinase. This observation suggests that the bacteria are affecting Src kinase expression that could be essential for its survival within the vector host. Src kinase is a protein that has an effect on the signal transduction pathway. This study suggests that tick-borne pathogens could manipulate Src kinase-mediated signal transduction events that are critical for its survival in ticks and tick cells. Figure 3C shows that the same result occurs on the protein level as well as at the transcript level. The observation of fainter band at about 60 kDa in Figure in the infected tick sample indicates that the production of the Src kinase protein decreases upon *A. phagocytophilum* infection. Figure 3D shows that during feeding the trend still shows a decrease in Src kinase expression and is still significant but less of a significance when compared to Figure 3B. These observations suggest that as the blood enters into tick there is a shift in the expression of Src kinase.

Figure 4A shows the result of larvae after they have finished a blood meal and it shows that there is now a significant upregulation occurring after the tick vector has received a blood meal from the host. The same trend can be seen in Figure 4B with nymphal ticks and this further helps reinforce the theory that there is a shift in Src protein levels during the blood meal

acquisition by ticks. Figure 4C also shows that the Src kinase protein amounts follow this trend and that there is an increase in the amount of Src kinase when the ticks are infected with *A. phagocytophilum*. Figure 3 shows data from unfed ticks and figure 4 shows data from fed ticks. The significant changes in Src kinase expression in fed ticks indicate that this protein kinase has an important role in *A. phagocytophilum* infection and survival in ticks and tick cells. In addition, upregulation of arthropod Src kinase expression upon tick feeding on *A. phagocytophilum*-infected mice suggests its role in transmission of this bacterium from vertebrate host to ticks. Collectively, the results from Figures 3 and 4 suggest that *A. phagocytophilum* modulate Src kinase expression to increase its infectivity, survival, and spread in ticks.

II. Tick *in vitro* analysis

Figure 5 shows the effect of different doses of *A. phagocytophilum* on Src expression in tick cells. This experiment was performed with different MOI to see the effects on Src kinase expression. Figure 5A shows that bacterial burden increased as the amount of isolated *A. phagocytophilum* solution was increased. This shows that the tick cells in each category had a different level of *A. phagocytophilum* burden depending on the dosage of solution added to each well. Figure 5B shows that the Src kinase expression in these tick cells was significantly increased at all tested doses. The observation of a significant change in Src expression between uninfected and infected tick cells suggests that there is an important biological role for this kinase in tick cells upon *A. phagocytophilum*-infection. During infection *A. phagocytophilum* increases Src kinase expression *in vitro* in order to increase its spread and infectivity. The difference between the uninfected and infected samples can also be seen with Figure 5C; the bands on the immunoblot gets darker with an increase in the amount of isolated *A. phagocytophilum* added to tick cells. This suggests that *A. phagocytophilum* modulate Src

kinase in tick cells to manipulate the signal transduction pathways critical for its survival. It is reasonable to hypothesize that *A. phagocytophilum* uses this Src kinase to influence signals inside the cell including affecting the expression of certain transduction factors important in cell regulation and apoptosis. With Figure 5, it can be noted that Src kinase plays a role in signal transduction pathways and is important for *A. phagocytophilum* survival in tick cells.

III. Tick dsRNA and inhibitor analysis

Figure 6 shows the impact of silencing and inhibition of Src kinase in ticks and tick cells. Figure 6A and 6B show that there is a difference in *src* transcript levels between the mock-treated and *src*-dsRNA-treated ticks. The observation of significant decrease in *src* transcripts expression in Figure 6A in *src*-dsRNA treated ticks in comparison to mock-treated ticks indicates good knockdown efficiency. Figure 6B shows that the *src*-dsRNA microinjected ticks have a decreased *A. phagocytophilum* burden in comparison to the burden noted in mock-treated ticks. There is a significant decrease in bacterial loads between the mock-treated ticks and *src*-dsRNA-treated ticks. This data shows that when Src kinase amounts and activity is reduced, it impacts the survival and spread of *A. phagocytophilum* in ticks. The observation of significant decrease in the amount of *A. phagocytophilum* burden in *src*-dsRNA ticks suggests that Src kinase plays a crucial role in *A. phagocytophilum*-tick interactions. The same trend can also be noted in tick cells in vitro as shown in Figures 6D and 6E. Figure 6D shows that the *src*-dsRNA-treated tick cells have a significant decrease in Src kinase expression when compared to the levels noted in mock-treated tick cells. This shows that the transfection was successful in tick cells and that lead to a decreased Src kinase expression. Figure 6E shows that when the tick cells were transfected with the *src*-dsRNA, a significant decrease in the detection of *A. phagocytophilum* was noted. This shows that silencing of *src* expression had a major impact on

the *A. phagocytophilum* burden within the tick cells. When Src kinase activity and production are inhibited by the treatment of tick cells with Src-inhibitor, a significant decrease in *A. phagocytophilum* burden was observed. The gene silencing experiments show that when expression of Src kinase is knockdown, a direct impact on the amount of *A. phagocytophilum* burden on the tick cells was observed. This indicates that this protein kinase plays a role with bacterial reproduction and spread. Figure 6C shows that when tick cells are infected, there is an increase in Src kinase expression. This result helps to reinforce the results from gene silencing experiments that indicates important role for Src kinase on bacteria survival in ticks. Figure 6F shows the effects of inhibiting Src kinase in tick cells. Upon Src kinase inhibition the level of *A. phagocytophilum* loads decreases. This shows that there is a decrease in *A. phagocytophilum* burden when tick cells were treated with the Src inhibitor. Collectively, the results from this study show the importance of Src kinase in *A. phagocytophilum* survival within ticks and tick cells.

CHAPTER V

CONCLUSIONS

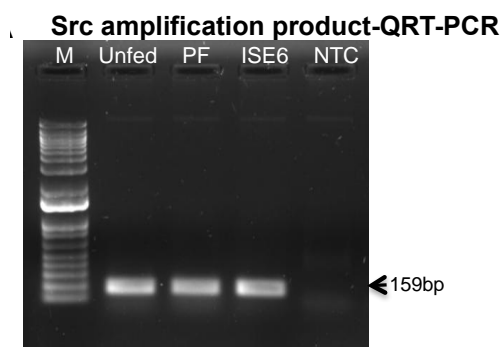
I. Src kinase conclusion

The findings from this study suggest that Src kinase plays an important role in cell signaling in ticks and tick cells. Src kinase is a protein that has effects on various pathways including cell cycle regulation, apoptosis, cell survival and host defense. With the data presented it shows that this protein kinase is manipulated by the bacteria *A. phagocytophilum* and this protein is necessary for its survival within ticks and tick cells. There are several proteins and kinases associated with cellular regulation, but the data shown implies that Src kinase has a large impact on the signal transduction pathway essential for *A. phagocytophilum* survival and infection of ticks and tick cells. Understanding how this protein kinase facilitates bacterial survival in ticks and tick cells could provide information for the development of strategies to target this and other rickettsial pathogens. Development of strategies to prevent the spread of this bacterium is very important. Creating a detailed signal transduction pathway modulated by this pathogen could give insights into the development of vaccine and ways to hinder and stop the spread and reproduction of *A. phagocytophilum* within ticks, tick cells and its transmission to vertebrate host.

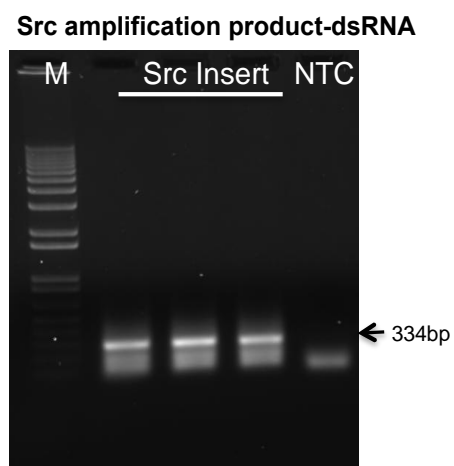
REFERENCES

- Alberdi, P., Espinosa, P. J., Cabezas-Cruz, A., and de la Fuente, J. (2016). *Anaplasma phagocytophilum* manipulates host cell apoptosis by different mechanisms to establish infection. *Vet. Sci.* 3, 15.
- Copiz, M. and Coloma, T. (2016). c-Src and its role in cystic fibrosis. *Euro. J. Cell Biol.* 95, 401-413.
- Ijdo, J., Carlson, A., and Kennedy, E. (2007). *Anaplasma phagocytophilum* Anka is tyrosinephosphorylated at EPIYA motifs and recruits SHP-1 during early infection. *Cell. Microbiol.* 9, 1284–1296.
- Khanal, S., Taank, V., Anderson, J., Sultana, H., and Neelakanta, G. (2018). Arthropod transcriptional activator protein-1 (AP-1) aids tick-rickettsial pathogen survival in cold. *Sci. Rep.* 8, 11409.
- Kocan, K., De la Fuente, J., and Cabezas-Cruz, A. (2015). The genus *Anaplasma*: new challenges after reclassification. *Rev. Sci. Tech. Off. Int. Epiz.* 34, 577-586.
- New, D.C., and Wong, Y.H. (2007). Molecular mechanisms mediating the G protein-coupled receptor regulation of cell cycle progression. *J. Mol. Signal.* 2, Art. 2.
- Sukumaran, B., Ogura, Y., Pedra, J. H. F., Kobayashi, K. S., Flavell, R. A., and Fikrig, E. (2012). Rip2 contributes to host defense against *Anaplasma phagocytophilum* infection. *FEMS Immunol. Med. Mic.* 66, 211–219.
- Sultana, H., Neelakanta, G., Kantor, F. S., Malawista, S. E., Fish, D., Montgomery, R. R., and Fikrig, E. (2010). *Anaplasma phagocytophilum* induces actin phosphorylation to selectively regulate gene transcription in *Ixodes scapularis* ticks. *J. Exp. Med.* 207, 1727–1743.

- Taank, V., Dutta, S., Dasgupta, A., Steeves, T., Fish, D., Anderson, J., Sultana, H., and Neelakanta, G. (2017). Human rickettsial pathogen modulates arthropod organic anion transporting polypeptide and tryptophan pathway for its survival in ticks. *Sci. Rep.* 7, 13256.
- Turck, J., Taank, V., Neelakanta, G., and Sultana, H. (2019). *Ixodes scapularis* Src tyrosine kinase facilitates *Anaplasma phagocytophilum* survival in its arthropod vector. *Ticks Tick-borne Dis.* 10, 838-847.
- Westhoff, M., Serrels, B., Fincham, B., Frame, C., and Carragher, N. (2004). Src-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol. Cell. Biol.* 24, 8113–8133.
- Wu, J., Weening, E. H., Faske, J. B., Höök, M., and Skare, J. T. (2011). Invasion of eukaryotic cells by *Borrelia burgdorferi* requires β_1 integrins and Src kinase activity. *Infect. Immun.* 79, 1338–1348.

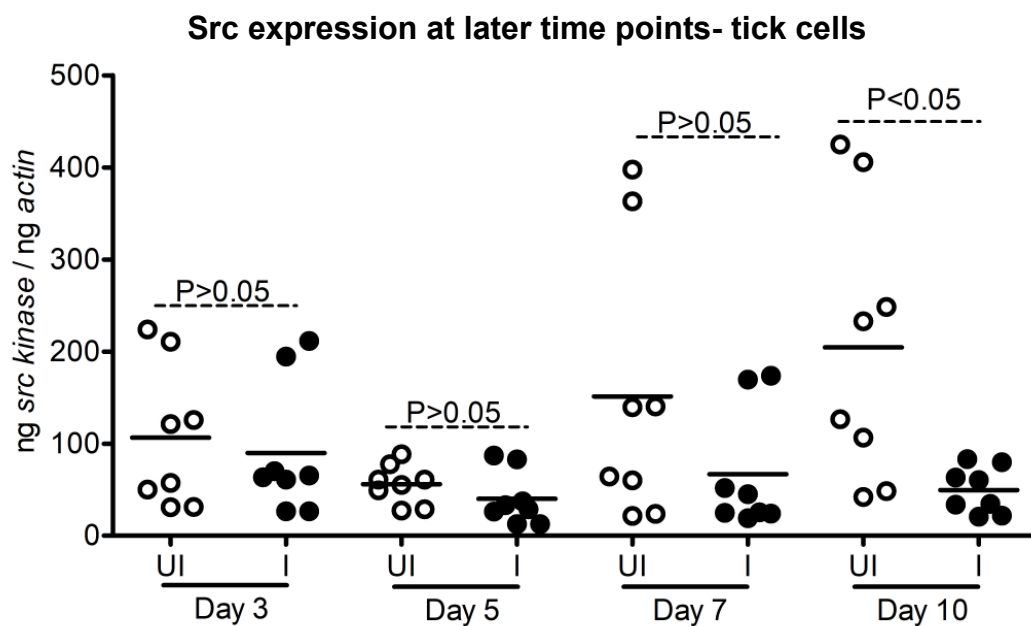
APPENDIX I. SUPPLEMENTARY FIGURE 1**Supplementary Figure 1. Gel Electrophoresis of the Src Kinase PCR Reactions.**

This figure represents gel electrophoresis experiment that was used to show the Src amplification product that occurred during the RT-qPCR reactions.

APPENDIX II. SUPPLEMENTARY FIGURE 2**Supplementary Figure 2. Gel Electrophoresis of the Src Kinase dsRNA Used for Clone Production.**

This figure represents gel electrophoresis experiment that was used to show the Src amplification that was used for the making of the dsRNA from the clone. This gel represents the Src kinase fragment that was used to combine with the L440 vector in order to create the clone.

APPENDIX III. SUPPLEMENTARY FIGURE 3



Supplementary Figure 3. In vitro Data for the Time Points for *Anaplasma phagocytophilum* Infection in ISE6 Tick Cells.

This figure represents the in vitro data that was used to show the Src kinase expression in tick cells during certain time points of infection with *Anaplasma phagocytophilum*.

APPENDIX IV. SUPPLEMENTARY INFORMATION

Supplementary Figure 1 shows the detection of the Src amplification product for the RT-qPCR experiments and the bands can be seen at 159 base pairs (bp). This figure shows a single amplicon using primers designed to amplify *src* kinase gene product. Supplementary Figure 2 shows the Src amplification product that is used for the dsRNA experiments. In this figure the bands for the Src kinase fragment can be seen in 334 bp. Supplementary Figure 3 shows the results of *A. phagocytophilum* infection in ISE6 tick cells at different time points. The results show a trend of decreased Src kinase expression but the data between the uninfected and infected samples is not significant. This trend follows the results of Figure 6C and show that there is a downregulation of Src upon *A. phagocytophilum* infection.

APPENDIX V. SUPPLEMENTARY ANALYSIS

The supplementary figures are used to show that there was proper amplification of the *src* kinase gene product that was used for both RT-qPCR experiments and gene expression silencing experiments. Supplementary Figure 1 shows amplification of a PCR product at 159 bp from different tick samples. NTC indicates no template control and reactions for NTC were performed with all PCR reagents except tick cDNA template. The dark band below the expected product size is a primer dimer that formed during the amplification process. Supplementary Figure 2 shows agarose gel image with amplification of *src* kinase gene product (334 bp) used for restriction digestion followed by cloning into pL4440 vector for making *src*-dsRNA. Primer dimers were also evident in the image and NTC indicates no template control. Supplementary Figure 3 shows *src* transcript levels in uninfected and *A. phagocytophilum*-infected tick cells at different time points post infection. This figure follows the same trend as Figure 4C but the data was not significant enough to show that there is a biological difference between the uninfected and infected tick cell samples.

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Publication: Molecular and Cellular Biology

Publisher: American Society for Microbiology

Date: Aug 30, 2004

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- Turck, J.W., Taank, V., Neelakanta, G., & Sultana, H. (2019). *Ixodes Scapularis* Src tyrosine kinase facilitates *Anaplasma phagocytophilum* survival in its arthropod vector. *Tick and Tick-borne diseases*, 10, 838-847. doi: 10.1016/j.ttbdis.2019.04.002

PRESENTATION

- Turck, J., Taank, V., Girish, N., and Sultana, H., *Ixodes scapularis* Src kinase is required for rickettsial pathogen survival.
 - Virginia Academy of Science (VAS) at Longwood University, 2018